The University of Chicago

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A PART OF A
DISSERTATION SUBMITTED TO THE FACULTY OF THE
DIVISION OF THE PHYSICAL SCIENCES IN CANDIDACY
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY
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FERMENTATIVE AND PHOTOCHEMICAL PRODUCTION OF HYDROGEN IN ALGAE

BY JACK RUBIN

(From the Department of Chemistry, The University of Chicago, Chicago)

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The investigation reported in this paper rendered mainly two results which are of importance for the analysis of photosynthesis in chlorophyllous plants. Firstly: Unicellular algae (*Scenedesmus* and similar species), which under anaerobic conditions are capable of reducing carbon dioxide with molecular hydrogen in the light, will liberate hydrogen slowly in the dark if air is replaced by nitrogen in the surrounding gas phase. This faculty for a hydrogen fermentation in *Scenedesmus* agrees with our knowledge of the dark metabolism of bacteria which utilize hydrogen. These reactions with hydrogen have often been found to be reversible. Secondly: Illumination of the fermenting algae enhances the liberation of hydrogen, particularly if the substrates of the photochemical reduction process, carbon dioxide and hydrogen, are both absent. The release of hydrogen by the algae under the influence of light occurs at a rate about ten times that of the hydrogen formation in the dark. The rate, however, is already limited at low intensities by factors other than the light intensity. Experiments with specific inhibitors, like dinitrophenol, allow us to differentiate between the dark and the photochemical liberation of hydrogen.

Upon return of the photosynthesizing cell to aerobic conditions both phenomena disappear as in the case of photoreduction.

Besides hydrogen and carbon dioxide, the anaerobic metabolism of *Scenedesmus* and of similar green algae includes many other substances, notably organic acids formed by fermentation of internal or artificially provided carbohydrates. As far as the nature of these fermentation products is concerned, the present investigation was extended only to the analysis of added glucose and to the identification and determination of lactic acid.

Methods

A description of the methods used in the present study has been reported elsewhere (Jack Rubin (27)), so that a few summarizing remarks will suffice.

Pure cultures of *Scenedesmus* species D_1, *S.* species D_3, and *S. obliquus* were grown at 20°C. in inorganic saline through which a slow stream of 4 per cent CO_2 in air was passed. The culture flasks, each containing 200 cc. of medium, were inoculated from an agar slant and illuminated for 3 to 4 days with incandescent lamps yielding about 4,000 lux at the bottom of the flasks.

The gas exchange of the algae was determined by Warburg's manometric method

(vessels with two side arms, inner well and vent stopper or with a side arm which can be closed and opened by turning the vessel).

In experiments requiring the addition of glucose bacterial contamination could be prevented for many hours if the manometer vessels were dried immediately before the experiment at 150°C. and if the algae were suspended in sterile sugar and buffer solutions. In a series of fifty experiments only two were found contaminated with bacteria. The pure nitrogen, and nitrogen mixtures, used to replace air were passed over finely divided copper at 500°. Hydrogen from the Ohio Chemical Company was used without further purification.

We used as light sources 1) a sodium lamp; its radiation as measured with a photronic cell calibrated against a thermopile is expressed in ergs/cm.^{2}/sec.; 2) several incandescent lamps. The intensity of illumination with these lamps is expressed in lux.

The identity and quantity of the gases involved in the metabolism of the algae have been determined with the familiar reagents introduced directly into the manometric vessels: carbon dioxide with potassium hydroxide solution; oxygen with alkaline pyrogallol; hydrogen by absorption with palladium black and decoloration of methylene blue in presence of platinum.

The total amount of organic acids formed in fermentation was measured by titration of the remaining bicarbonate in the medium. Glucose was determined by a modification of the Shaffer-Hartmann method (28); lactic acid was determined colorimetrically with *p*-hydroxydiphenyl, the intensity of absorption measured with a photocell (29).

PART I

The Formation of Hydrogen in the Dark

It is well known that green plants begin to ferment as soon as the oxygen partial pressure in the surrounding medium drops below the value necessary to maintain the Pasteur effect (inhibition of fermentation by oxygen). In the dark a suspension of algae in water or in an acid buffer will produce carbon dioxide when placed in an atmosphere of nitrogen. If we place the algal suspension in a Warburg vessel, the evolution of carbon dioxide can be followed manometrically. With a 5 per cent solution of potassium hydroxide in a side arm of the vessel, no pressure changes are visible. This indicates that no other gas is liberated in fermentation but carbon dioxide. All this is common knowledge. With several strains of the algae *Scenedesmus* we observed, however, that under the conditions described a Warburg manometer containing a potassium hydroxide solution in the side arm showed the development of a gas different from carbon dioxide. A comparison between identical samples of a *Scenedesmus* suspension fermenting in absence and in presence of an absorbent for carbon dioxide revealed that the new gas begins to appear after a prolonged anaerobic incubation of about 2 hours at 25°. This gas is formed in addition to, and independent of, the evolution of carbon dioxide which is liberated from the very beginning.

Fig. 8[1] demonstrates the course of the anaerobic gas production by *Scenedesmus* in absence (curve *a*) and presence (curve *b*) of potassium hydroxide. The vessels used were of equal volume (15.4 and 15.0 cc.) The broken line is obtained as the difference between curves *a* and *b*. It indicates that curve *a* would continue as a straight line except for the additional production of gas beginning after 2 hours of fermentation. Experimentally the rate of fermentation is found to continue unchanged when the vessel showing normal fermentation contains a sufficient amount of palladium black.

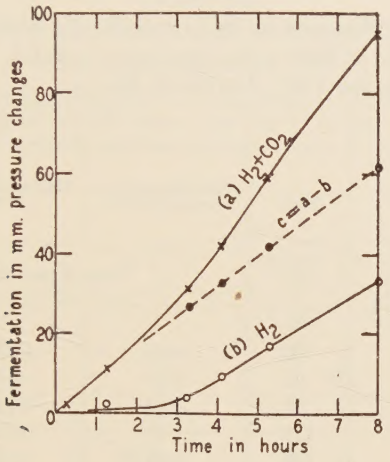

FIG. 8. Liberation of molecular hydrogen during fermentation in the alga *Scenedesmus*. 0.027 cc. of cells in culture medium with 0.01 M phosphate buffer pH 6 containing 0.2 per cent glucose. Temperature 25°. Curve (*b*): KOH solution in side arm of vessel, absorbing CO_2.

The appearance in *Scenedesmus* of a new metabolic reaction after a fermentation period of 1 or 2 hours at 25° agrees with the observations on the photoreduction with hydrogen. The latter becomes possible in *Scenedesmus* not simply by substituting hydrogen for air, but only after a suitable anaerobic incubation period.

This similarity suggested that the same system, which in its reduced state enables the cells of *Scenedesmus* to utilize hydrogen, was also responsible for the production of the gas not absorbed by potassium hydroxide. The data contained in Table X are, in our opinion, evidence that this gas is hydrogen. If equal amounts of *Scenedesmus* cells fermenting in the same medium are compared, the difference in the amount of gas produced in absence and in presence of palladium black is equal to the gas production found in presence of potassium hydroxide.

[1] Figs. 1–7 and Tables I–IX belong to the preceding paper (I).

Qualitatively it can be shown that the gas decolorizes a methylene blue solution in contact with platinum.

The gas is not nitrogen, methane or ethylene because none of these three substances will react anaerobically with palladium in the way observed. It is not oxygen because it is not absorbed by alkaline pyrogallol. Nor is it carbon monoxide because we have found carbon monoxide to be a specific inhibitor for this reaction and all metabolic reactions in *Scenedesmus* involving molecular hydrogen.[2]

TABLE X

Production of Hydrogen during the Fermentation of Scenedesmus (Species D_1)

0.095 cc. of cells in 3 cc. of 0.025 M phosphate buffer. pH 6.2. Suspension contains 0.07 per cent glucose. Temperature: 36°. Gas phase: N_2.

	Time	1	2	3
		CO_2 measured in presence of Pd	H_2 measured over KOH	H_2 measured as difference between pressure changes in vessels with and without Pd
		Rates of gas exchange in $\frac{c.mm.}{30\ min.}$		
	min.			
Dark (after 40 min.)	30	+28	+10.4	+8.1
	60	+20	+7.5	+10.5
	90	+24	+10.4	+7.2
	170	+21	+7.9	+7.9
	200	+18	+4.5	+5.9
Light, λ5890Å				
$I = 3 \times 10^3$ ergs/cm.²/sec.	30	+20	+9	+5.5
$I = 12 \times 10^3$ ergs/cm.²/sec.	120	+13 → +27	+22 → +25	+0.3 → −7.5*

* In the light the method used for the data in column 3 ceases to be useful.

Autofermentation.—In order to observe a measurable fermentation it is not necessary to add a substrate to the cell suspension. The source of hydrogen is one of several unknown reserve substances stored in the cell. The rate of the fermentative evolution of hydrogen in *Scenedesmus* declines with time. Starved cells, which have been allowed to respire for a long time in the dark, show a low rate of fermentation and of hydrogen formation with a tendency toward a further decline with time. Cells which have assimilated carbon dioxide in the light for several hours give the best yield of autofermentation including the liberation of hydrogen.

[2] Since the manometric hydrogen determination is accurate only to about 5 per cent, there is ample space for the discovery that still another gas is formed in traces during fermentation.

Dependence of the Evolution of Hydrogen on the Hydrogen Partial Pressure.— In the experiments on photoreduction or on the oxyhydrogen reaction the algae were incubated not in nitrogen but in hydrogen for a period of several hours previous to the experiments. During this time they invariably absorbed hydrogen sometimes in considerable amounts. Not only in presence of an absorbent for carbon dioxide, but also if the carbon dioxide of fermentation was allowed to accumulate, the balance of the overall gas exchange was negative. The algae fermenting in hydrogen absorb more gas than they produce.

TABLE XI*a*

Fermentation of Scenedesmus obliquus in Hydrogen and in Nitrogen

0.105 cc. of cells in 3 cc. of 0.05 M phosphate buffer. pH 6.2. 0.4 cc. of 2 per cent KOH solution on a piece of paper in the side arm of the vessels. Turning the vessels around their vertical axis closes or opens the side arms. (See Fig. 14 in Paper III.) Temperature: 25°. 2 hours of fermentation in N_2 in both vessels before start of experiment. Vessel 1 filled with H_2.

		1		2	
		Side arm closed	Side arm open	Side arm closed	Side arm open
Vessel constant for CO_2		1.49	1.74	1.56	1.83
Vessel constant for H_2		1.25		1.32	
		H_2		N_2	
	Time	Pressure changes in mm.			
Dark	12 hrs.	−81		+112	
	30 min.		−73		−71

Computation of Gas Exchange

In H_2	*In N_2*
$73 \times 1.74 = +\ 127$ *c.mm.* CO_2	$71 \times 1.83 = +\ 130$ *c.mm.* CO_2
$\left(-81 - \frac{127}{1.49}\right) \times 1.25 = -\ 207$ *c.mm.* H_2	$\left(112 - \frac{130}{1.56}\right) \times 1.32 = +\ 37$ *c.mm.* H_2

If we compare this with the opposite results on the fermentation in nitrogen, it is obvious that the presence or absence of hydrogen in the gas phase must influence the activity of the hydrogenase system.

Table XI*a* and *b* presents the results of an experiment in which algae of the same culture were brought into two vessels, and one part was left to ferment in pure nitrogen, the other part in pure hydrogen. To make sure that conditions were comparable in both vessels the hydrogen was introduced into one vessel after a preliminary fermentation period of 2 hours in nitrogen during which the gas exchange in both vessels was equal. The design of the vessels used (described in Paper III) is such that after a certain time a side arm containing potassium hydroxide is opened. In this manner the carbon dioxide released from the slightly acid algal suspension during the interval is measured and compared with the total gas exchange.

In this particular experiment the amount of carbon dioxide formed under nitrogen was equal to that liberated under hydrogen. Simultaneously the algae in nitrogen had produced hydrogen in a quantity equal to about one-third the amount of carbon dioxide formed while the algae in hydrogen had absorbed hydrogen in an amount about twice that of the carbon dioxide. There are as yet no experiments available which allow one to decide whether a high partial pressure of hydrogen reverses the process of hydrogen production or whether both liberation and absorption proceed simultaneously and independently. A parallel experiment with algae suspended in bicarbonate showed that the fermentative formation of acids was the same in hydrogen as in nitrogen (Table XI*b*). The first possibility, the existence of a true equilibrium between bound and free hydrogen, would offer an explanation for the

TABLE XI*b*

Acid Formation during Fermentation in Hydrogen and in Nitrogen

0.114 cc. of cells of *Scenedesmus* D_1 in 3 cc. of 3.3×10^{-2}M $KHCO_3$. Temperature: 25°. 40 minutes fermentation in N_2 before experiment.

No. and volume of vessels	(1) 18.6 cc.	(2) 18.0 cc.	(3) 18.4 cc.	(4) 18.2 cc.
Initial bicarbonate content in each vessel 212 c.mm. CO_2				
Gas phase	H_2; 4 per cent CO_2		N_2; 4 per cent CO_2	
Total pressure change in 15 hrs., *mm*	−94	−94	+167	+176
Final bicarbonate content in c.mm. CO_2	114	117	116	116
Acid formed in H_2 and N_2	*96 c.mm.*		*96 c.mm.*	

fact that sometimes the determination of hydrogen formation by difference in presence and absence of palladium renders results not checking with the direct determination of hydrogen formed in presence of alkali. The accumulation of hydrogen in the vessels without palladium might gradually inhibit the evolution of hydrogen—which proceeds uninhibited where the hydrogen is continuously removed.

Influence of Glucose and of Other Organic Substances upon the Hydrogen Formation.—Of many organic substances added to the cell suspension only glucose was found to increase immediately all phases of the anaerobic metabolism (Table XII). Other monosaccharides were effective after a definite time lag. The phosphates of glucose (mono- and di-), of glycerol, of glyceric acid, etc., were all without effect. They probably did not penetrate to the proper places. Pyruvic and lactic acids gave positive results, but the difficulties, with respect to permeability and to the inhibiting properties of these acids, leave it an open question whether their effect is in any way to be looked upon as a direct one.

The experiments shown in Fig. 9 and in Tables XII–XIV demonstrate the accelerating effect of added glucose. As yet we have found no evidence that formate could be an intermediate in the course of the hydrogen production. (Compare reference 25, page 96.)

Influence of the Hydrogen Ion Concentration.—Contrary to the behavior of respiration and photosynthesis, the rate of fermentation in the algae is clearly dependent on the hydrogen ion concentration in the suspension. The optimal hydrogen ion concentration varies with other conditions influencing the fermentation, *e.g.* the absence or presence of added glucose and the

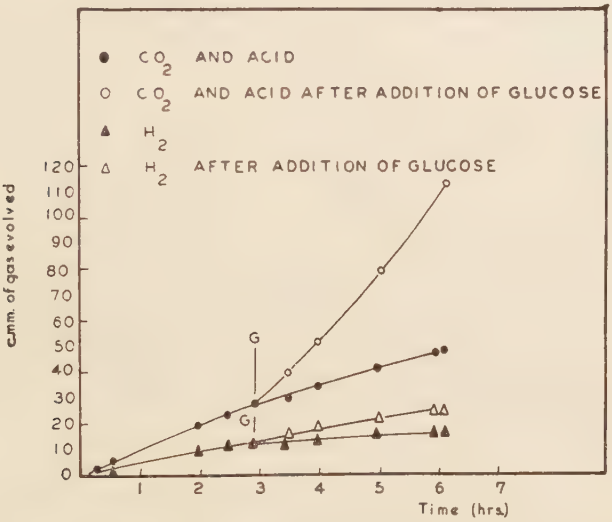

FIG. 9. Increase of fermentation and of hydrogen production in *Scenedesmus* upon addition of glucose. Final sugar concentration 0.06 per cent.

time elapsed since the beginning of the anaerobic period. (See Fig. 10.) Such variations of pH optima are already known in bacterial metabolism (25). For our purposes a pH between 6 and 7 appeared best.

The Relative Amounts of Carbonic and Organic Acids Produced in Fermentation.—Whereas it could be established beyond doubt that added glucose enhances the production of hydrogen, numerous experiments failed to reveal a clear and uniform relationship between the amounts of the different fermentation products and of the glucose added. The hydrogen production remains rather small compared with the quantities of potential hydrogen donors available. (See Table XIV.)

There is also hardly a constant relation between the volume of hydrogen evolved and that of carbon dioxide gas or of fixed acids produced during a given fermentation period (Tables XI, XII, XIV, and XV). This is not too

TABLE XII

Influence of Glucose on the Hydrogen Fermentation

0.048 cc. of cells of *Scenedesmus* D_1 in 3 cc. of 0.01 M $KHCO_3$. Gas phase: N_2; 4 per cent CO_2. Temperature: 36°. Gas exchange computed from experiments in presence and absence of palladium black, which absorbs all hydrogen. The data represent the average of three (six) parallel experiments.

	Time elapsed since start of experiment	Rate of gas produced in $\frac{\text{c.mm.}}{30 \text{ min.}}$		Ratio $\frac{CO_2}{H_2}$
		H_2	CO_2	
	min			
(*a*) Autofermentation	70	3.5	7.8	2.2
	100	3.0	5.5	1.8
	130	2.0	6.6	3.0
(*b*) Glucose added (0.1 per cent)	160	4.5	11.3	2.5
	220	10.7	18.2	1.7
	280	6.3	18.6	2.9
	400	4.6	14.4	3.1

TABLE XIII

Influence of Carbohydrates on the Rate of Hydrogen Production

0.05 cc. of cells of *Scenedesmus obliquus* in 4 cc. of 0.05 M phosphate buffer. pH 6.5. Different sugars added aerobically. Final concentration in medium: 0.12 per cent. Side arms of all vessels contain 0.2 cc. of potassium hydroxide-pyrogallol solution. Temperature: 25°. Gas phase: N_2.

	Time	Control	Glucose	Galactose	Mannose	Hexosedi- and hexose mono-phosphate
		Rate of hydrogen production in $\frac{\text{c.mm.}}{10 \text{ min.}}$				
Dark	140 min.	0.7	1.2	0.7	1.0	0.6
Light. 3700 lux	10 min.	6	10	7	7	8
Dark	11 hrs.	0.3	0.7	0.7	0.7	0.3
Dark	20 min.	All vessels flushed with N_2 to remove H_2 of fermentation				
Light. 1700 lux	5 min.	6	18	10	12	8
Light. 1700 lux	15 min.	4	11	5	5	4

surprising if we remember that the hydrogen fermentation needs some additional adaptation before it can start, as compared with the ordinary fermentation which is regulated simply by the oxygen partial pressure (Pasteur effect). It is very probable that two or more independent fermentation

TABLE XIV

Acid Formation during Fermentation of Scenedesmus (Strain D_1)

0.06 cc. of cells in 3 cc. of 0.5×10^{-2}M $KHCO_3$. Temperature: 36°. Gas phase: N_2; 4 per cent CO_2. Duration of experiment 5 hours, after 1 hour of preliminary anaerobic incubation. All data expressed in cubic millimeters normal gas.

	Initial	Final	Difference	Initial	Final	Difference
(*a*) Glucose expressed in c. mm. normal gas (180 mg. equivalent 22400 c.mm.)	—	—	—	Filled in: 620, found: 581	361; 344	229
(*b*) Acid formed = change in bicarbonate	201; 205	110; 114	91	203	59; 59	144
(*c*) Lactic acid formed	3.6	5.3; 7.2	2.6	3.6	31; 33	28.4
(*d*) Total gas formed ($H_2 + CO_2 +$ decomposed bicarbonate)	0.0	175; 175	175	0.0	291; 297	294

Adding glucose results in the production of 53 c.mm. more organic acid of which 25 c.mm. = 50 per cent are lactic acid. Only 3 per cent of the 91 c.mm. acid formed in fermentation of intracellular material are lactic acid.

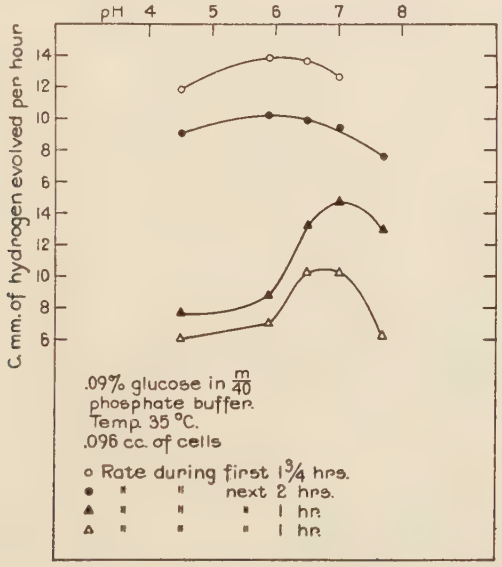

FIG. 10. Influence of the hydrogen ion concentration in the medium upon the rate of hydrogen liberation in the dark.

reactions can proceed simultaneously in *Scenedesmus*, perhaps, at quite different locations in the cell. We shall demonstrate in Part II that the hydrogen-liberating system may be linked with the photochemical activity of the plants, and the latter has its seat in the chloroplasts. On the other hand, it is known that the non-chlorophyllous parts of a plant have a definite fermentative capacity of their own. The situation is similar to that found with respiration in *Chlorella*. Autorespiration of photosynthetic products has different properties from that of substrates artificially supplied (Emerson (30)). As it was (13) pointed out before, one should consider in such a case the possibility

TABLE XV

Effect of Light upon the Liberation of Molecular Hydrogen in the Alga Scenedesmus

Algae grown with 1 per cent glucose. Suspended in phosphate buffer pH 6.5. Gas phase N_2 (free of CO_2). Temperature: 25°. Preceding dark time: 12 hours.

No.	1	2	3	4
Volume of vessels, *cc.*	18.3	18.0	18.4	18.2
Volume of liquids, *cc.*	3.0	3.2	3.0	3.2
In side arms	—	KOH	Pd	KOH, Pd
	Rate of pressure changes in $\frac{mm.}{hr.}$			
Dark	+16	+2	+13	+0.3
Light. 740 lux	+31	+15	+18	+2
Light minus dark	+15	+15	+2	+1
Dark	+17	−2	+18	+1
Light. 1500 lux	+21	+15	+18	0
Light minus dark	+4	+17	0	−1

not only of different catalytic systems but also of a separate location in the cell of these systems.

In some experiments we tried to shorten the adaptation time and to increase the rates by raising the temperature from 25 to 35°. Although fermentation continues at the higher temperature for many hours the photosynthetic capacities of the cells suffer an irreversible damage. Such cell samples should not be used in combined dark and light experiments. One might use inhibitors to separate and isolate the different fermentation processes from one another. Kempner, for instance, detected that the hydrogen fermentation of *B. butyricum* was stopped by carbon monoxide (26). Later Kubowitz (31) showed that this effect is not a true inhibition of glucose decomposition but a change from hydrogen fermentation to lactic acid formation.

Our experiments with inhibitors reported below gave results resembling those of Kempner and Kubowitz with butyric acid bacteria. Since in *Scene-*

desmus at least two types of fermentation reactions seem to occur simultaneously in varying proportions, more work than we have devoted to this problem is needed to disentangle the fermentation reaction and to establish the stoichiometry of the reaction yielding hydrogen.

The Formation of Lactic Acid.—Besides other acid compounds of yet unknown constitution, lactic acid appears only in very small amounts during autofermentation of natural reserve material, as shown in Table XIV. On the other hand, this acid often accounts for 50 per cent of the fermentation acids formed after adding glucose to a cell suspension. Barker found in the colorless heterotrophic alga *Prototheca* that glucose is decomposed up to 95 per cent into lactic acid.

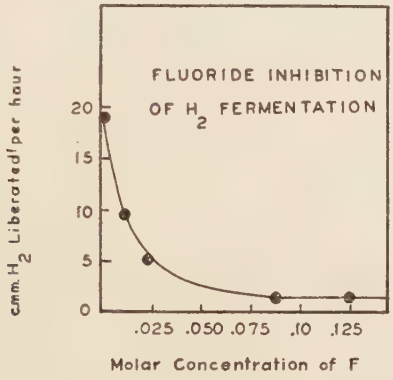

FIG. 11. Inhibition of hydrogen production by fluoride. Abscissa: molarity of fluoride ion.

Attempts to inhibit only the lactic acid formation with fluoride were not successful. The dark formation of hydrogen is also sensitive to fluoride (Fig. 11).

Q_{H_2} (*Dark*).—The familiar quotient Q_{H_2} (dark) $= \frac{\text{c.mm. } H_2}{\text{hrs.} \times \text{mg. dry cells}}$ was found to attain values not larger than 1. The weight of the dry material in milligrams was assumed (according to earlier measurements) to be 1/5 of the number of cubic millimeters of wet cells tightly packed by 15 minutes centrifugation at 3,000 rev./min.

CONCLUSIONS

From the evidence presented above, we have to conclude that those algae which have the capacity for photoreduction with hydrogen contain a hydrogenase capable of functioning independently of any photochemical process. Hydrogenase functions here as a catalyst establishing an equilibrium between

molecular hydrogen and various intracellular hydrogen-transferring systems. The potential of these hydrogen-transferring systems and the partial pressure of free hydrogen determine whether hydrogen will enter or leave the algal cell. (Compare Farkas (33).)

PART II

The Photochemical Production of Hydrogen

Under the proper anaerobic conditions, *Scenedesmus* and similar algae can utilize hydrogen for the reduction of carbon dioxide. We expected, therefore, that the hydrogen production by fermentation in the dark would disappear completely upon illumination. This is true indeed if carbon dioxide is present in sufficient amounts, either from the beginning of the experiment or if it accumulates during fermentation periods. When the algae are irradiated, hydrogen fermentation gives way to photoreduction and eventually to normal photosynthesis. Shape and duration of these transitions depend on the intensity applied and the conditions of the plants (see Paper I).

In the absence of carbon dioxide (*e.g.* KOH in side arm of the manometer vessel), the irradiated cells can make use only of that small part of the carbon dioxide formed in fermentation which does not escape into the gas phase. In the light, therefore, there will be neither an appreciable photoreduction nor a return to aerobic conditions (which depends on the presence of sufficient carbon dioxide), nor any photo-oxidation (which depends on available oxygen). Under these conditions light absorbed by the cells might be transformed into heat. With *Chlorella*, for instance, no observations have been made which would contradict such an assumption. The situation is surprisingly different, however, with the algae possessing a hydrogenase system. Irradiating *Scenedesmus* under anaerobic conditions ("reduced state") in absence of carbon dioxide, we observed a measurable photochemical activity. This new reaction is a photochemical production of gas which continues for hours, though at a rapidly declining rate. The gas liberated under the influence of light is hydrogen, and the algae produce it at a rate up to ten times the dark fermentation (Tables XV, X–XIII, XVI).

The evidence for the gas to be hydrogen is the same as already discussed in Part I. The gas is not absorbed by alkali, nor alkaline pyrogallol, which remains colorless (Table XVI). The decisive test that the gas produced under the conditions described is not the oxygen of photosynthesis is furnished by the photochemical reaction following the introduction of a mixture of hydrogen and carbon dioxide into the vessel containing the irradiated algae. If the gas produced photochemically by the algae were the oxygen of normal photosynthesis, they would continue to produce oxygen, probably at a much higher rate. What actually happens is that the algae cease to produce gas and turn to photoreduction with hydrogen without requiring the usual "adaptation

period" (Fig. 12). This is proof that the photochemical mechanism in these organisms has remained in the "reduced state" where no liberation of oxygen occurs. Furthermore, the gas in question is completely absorbed by palladium black (Table XV).

In an atmosphere of hydrogen, the photochemical evolution of hydrogen (in absence of the reducible substrate CO_2) is inhibited as compared with the reaction in nitrogen. Hydrogen is often released from the cells, however, in spite of the presence of a little hydrogen and carbon dioxide. Fig. 12 shows that whether we observe photoreduction or hydrogen evolution depends on

TABLE XVI

Photochemical Development of Hydrogen in Scenedesmus

0.04 cc. of cells in 0.05 M phosphate buffer. pH 6.5. Temperature: 38°. Gas phase: N_2. In side arms of vessels 0.2 cc. of 7 per cent KOH solution.

	1	2	3
Volume of vessels, *cc.*	16.0	15.0	15.4
	Pressure changes in mm.		
Dark, 240 min.	+27	+41	+30
Light, 240 min. 1000 lux	+74	+66	+66
0.1 cc. of a concentrated pyrogallol solution added to KOH solution in side arm	No color	Trace yellow	No color
	No significant pressure changes		
O_2 introduced into vessels, *mm.*	30	75	50
Color change in side arm 5 min. later	Yellow	Brown	Yellow
Gas absorbed in the dark during first 15 min. after O_2 has been introduced, *mm.*	−9	−26	−17

the light intensity. In the experiments, one of which is represented in Fig. 12, the algae had been fermenting for several hours in nitrogen. About 0.2 vol. per cent of hydrogen had accumulated on account of this fermentation. All carbon dioxide diffusing into the gas phase had continuously been absorbed by potassium hydroxide, while it was allowed to accumulate in a parallel experiment not shown here. Only during the last phase, where some hydrogen had been introduced into the vessels, a difference between the reaction of both samples of cells was found. This consisted in a less rapid decline of the rate of photoreduction in the vessel which had no potassium hydroxide solution in its side arm. We learn from the time course of the gas exchange in Fig. 12 that an absorption of hydrogen at very low light intensities can change instantaneously into a liberation of hydrogen at higher light intensities. For 20 minutes a Q_{H_2} of + 4 is maintained. That this evolution of gas is not a

turnback to photosynthesis (which is not likely to occur in absence of carbon dioxide) is demonstrated by the photochemical absorption of added hydrogen immediately afterwards.

The Influence of Hydrogen Donors.—The photochemical evolution of hydrogen depends clearly upon the presence of suitable hydrogen donors in the cell. The same circumstances which improve the dark hydrogen fermentation, addition of glucose or a previous intense photosynthesis, increase the yield of

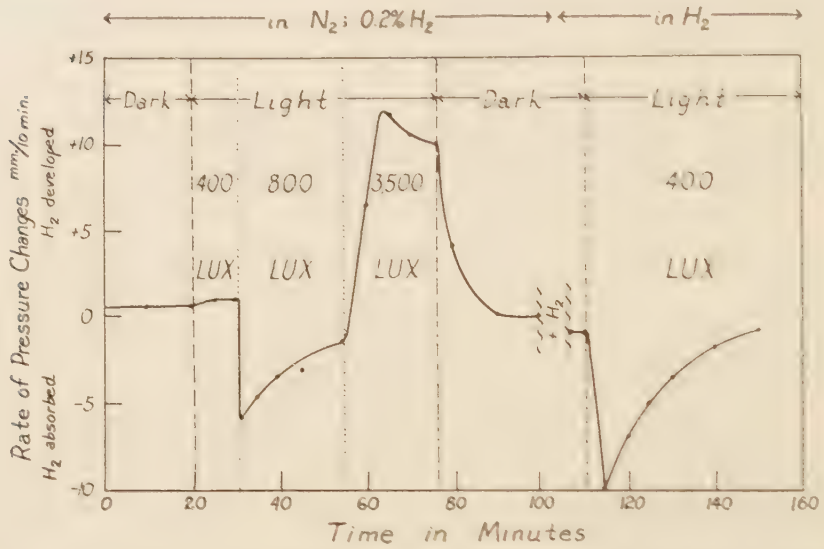

FIG. 12. Rates of photochemical absorption and evolution of hydrogen in absence of carbon dioxide at different intensities. 0.074 cc. of cells of *Scenedesmus* D_3 in 0.05 M phosphate buffer pH 6.2. Side arm of vessel contains KOH. Gas phase: N_2 later H_2. Preceding dark period: 20 hours in N_2. During this time the algae have formed H_2 amounting to 0.2 per cent of the available gas volume. Temperature: 25°.

the photochemical hydrogen production. The rate decreases with time, and we have often observed an apparent exhaustion of the supply of hydrogen donors. This indicates that dark reactions constitute factors limiting the overall rate of the process.

In spite of the fact that the same conditions which favor the hydrogen fermentation also support the photochemical evolution of hydrogen we must emphasize that the new light reaction is not simply an acceleration of the dark fermentation which continues unchanged. Table XV contains data which make this fact clear. It is important for the understanding of the photochemical reaction that the effect of light is apparently restricted to an increase of the production of hydrogen alone, and does not increase simultaneously the

output of free carbon dioxide and of organic acids. This will become even more apparent in Part III concerning the effect of poisons on the photochemical hydrogen production.

Anaerobic Induction Period.—The tendency of the algae to liberate hydrogen upon illumination after an anaerobic fermentation period prevails even in presence of carbon dioxide, though only for a short time. Gaffron (18, 20) has described manipulation of the gas exchange of illuminated algae preceding normal photosynthesis when the plants had been kept for several hours in nitrogen. With algae of the *Scenedesmus* type, the gas exchange during the anaerobic induction period consists first of a vigorous evolution of gas lasting from 1 to 3 minutes followed by a gas absorption which then turns into normal photosynthesis. (Compare Figs. 1 and 2 in reference 20.) Since at that time it was found that no oxygen is formed during the first two phases of the anaerobic induction period, and since Blinks and Skow, working with other species of algae, reported an initial carbon dioxide gush during the first seconds of illumination, Gaffron interpreted the first momentary gas production as a liberation of carbon dioxide. We have repeated these experiments in the presence of palladium black. In all cases the greater part, and in some cases all of the initial gas evolution disappeared in presence of this absorbent for hydrogen, while the second and third phase of the anaerobic induction period remained more or less as described. In our opinion the correct interpretation of the anaerobic induction period with algae containing a hydrogenase system is as follows: After a fermentation period of many hours the reduction of carbon dioxide is initially inhibited, possibly on account of organic acids, and the plants react as if carbonic acid were absent. That is, they start to produce hydrogen. With the beginning of carbon dioxide reduction, the evolution of hydrogen ceases and a period of photoreduction with internal hydrogen donors follows. Then, depending on the light intensity used, this is succeeded more or less quickly by normal photosynthesis with the evolution of oxygen. (Compare Fig. 13*a* with Fig. 13*b* and with Fig. 1 in reference 20.) According to the recent results of Emerson and Lewis with *Chlorella* (32), some carbon dioxide is released during the aerobic induction period upon illumination of the algae after a longer dark pause. How much CO_2 is liberated before normal photosynthesis starts depends on the condition of the algae and the concentration of carbon dioxide in the surrounding medium. It is possible, therefore, that the initial evolution of hydrogen during the anaerobic induction period in *Scenedesmus* is accompanied in the same way by varying amounts of carbon dioxide.

Q_{H_2} (*Light*).—If we express the rate in $\frac{\text{c.mm. } H_2}{\text{hrs.} \times \text{mg. dry weight}}$ generally a value of 3 and a maximal value of 7 is obtained. Though the hydrogen liberation in the light is about ten times the rate in the dark (with very large deviations to both sides), it is still very small as compared with the saturation rate

of normal photosynthesis. The rate of photoreduction with hydrogen seldom surpasses the order of magnitude of respiration in the same algae, and the

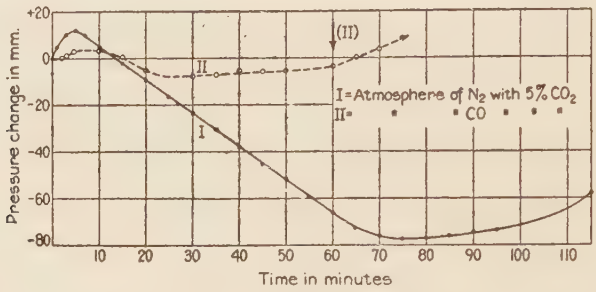

FIG. 13*a*

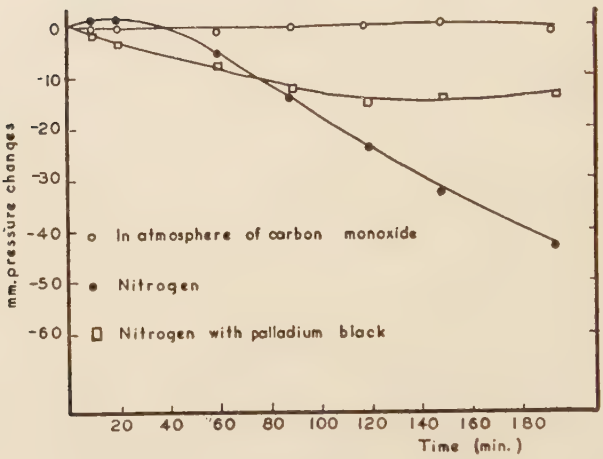

FIG. 13*b*

FIG. 13. Gas exchange of *Scenedesmus* during the induction period after several hours of anaerobic incubation in N_2 and its inhibition by carbon monoxide. Fig. 13*a*, according to Gaffron in 1935. The initial evolution of gas was interpreted as an "oxygen gush," followed by an internal CO_2 reduction. Fig. 13*b* shows a similar experiment. In presence of palladium black the initial gas production is not visible. The rate of photoreduction appears to be smaller because only intracellular hydrogen donors are available. The hydrogen developed during the preceding fermentation period has been absorbed by palladium. All anaerobic photochemical gas exchange is inhibited by carbon monoxide.

photochemical hydrogen evolution stays mostly below those rates. It appears, from studies of the oxyhydrogen reaction in the dark (Paper III), that in all cases the hydrogen transfer systems (including the hydrogenase) are responsible for these slow rates.

PART III

The Effect of Specific Inhibitors upon the Production of Hydrogen in Algae

The preceding observations on the hydrogen fermentation and on the photochemical evolution of hydrogen in green algae of the *Scenedesmus* type indicate that both reactions, though enhanced by the same anaerobic conditions, may have only one catalyst in common and are independent of one another in other respects. In this part, we present more evidence supporting the assumption that the hydrogen liberated photochemically may have a different origin than that developed by fermentation. With dinitrophenol as a poison, it is possible to inhibit largely if not completely the evolution of hydrogen in the dark while the photochemical production of this gas continues at the normal or even an increased rate (Tables XVII–XIX).

As described in the preceding paper (Paper I), minute amounts of dinitrophenol influence all phases of the algal metabolism. In agreement with results obtained with other organisms reported in the literature, the inhibiting or stimulating effect is a function not only of the total concentration of dinitrophenol, but also of the hydrogen ion concentration, probably because the free acidic form is the poison proper. Table XVIII shows that 4×10^{-4} M dinitrophenol at pH 6.2 inhibits the dark hydrogen fermentation, while at the same time the lactic acid formation is increased. (See also Table XIX.) This appears to be a metabolic change similar to that known to occur in *B. butyricum* (Kempner-Kubowitz (26); (31)) where the inhibition of the hydrogen liberation by cyanide or carbon monoxide leads to an increase in lactic acid production. The stoichiometric relations, however, are different inasmuch as the uninhibited hydrogen fermentation in algae is already a smaller fraction of the total anaerobic metabolism than in the bacteria mentioned.

Table XIX shows what happens upon illumination of the poisoned algae in comparison with those kept under normal anaerobic conditions. As in all experiments on photosynthesis, etc., we assume the true effect of light to be equal to the difference between the metabolic rates in the light and in the dark. An essential difference of this experiment from that presented in Table XV is that the gas phase contains 4 per cent of carbon dioxide in nitrogen instead of pure nitrogen. The unpoisoned algae will respond to light, therefore, with a photoreduction of carbon dioxide, partly at the expense of the hydrogen developed during the previous dark time, partly or mainly at that of the internal hydrogen donors. The pair of manometer vessels containing the poisoned algae present a quite different result. The light effect here is the production of gas which is completely absorbed by palladium; *i.e.*, a production of hydrogen. Hydrogen is liberated photochemically though its fermentative production has been oppressed by the poison. Furthermore, hydrogen appears despite the presence of carbon dioxide. The action of the poison is thus two-

fold. Firstly, it differentiates between the mechanisms of hydrogen production in the dark and in the light. Secondly, it inhibits the photoreduction

TABLE XVII

Effect of Dinitrophenol on the Production of Hydrogen in Scenedesmus

(*a*) 0.050 cc. of cells in 3 cc. of 0.05 M phosphate buffer. pH 6.2. Temperature: 25°. Gas phase: N_2. In the side arm of the vessels 0.2 cc. 7 per cent KOH. Light intensity I (sodium line 5890 Å) measured in 10^3 ergs/cm.^{2}/sec.

		1	2
Volume of vessels, *cc.*		19.8	21.2
Condition	Observation time	Rate of H_2 evolution in $\frac{\text{c.mm.}}{\text{10 min.}}$	
	min.		
Dark	120	+0.85	+ 0.70
Poison added	—	—	2×10^{-4} M dinitrophenol
Dark	90	+1.03	±0
Light I = 3.0	50	+1.8	+1.9
Dark	80	+0.4	(−0.2)
Light I = 7.5	30	+6.1	+8.1
Light I = 12	15	+9.1	+11.0

(*b*) 0.04 cc. of cells of *Scenedesmus* D_3 in 4 cc. of 0.033 M phosphate buffer. pH 6.3. In Experiments 2 and 3 the suspension contains 5 mg. glucose. Temperature: 25°. Gas phase: N_2 (control H_2; 4 per cent CO_2). Preceding incubation time: 3 hours.

		1	2	3
Volume of vessels, *cc.*		15.0	16.0	15.4
Gas phase		H_2/CO_2	N_2	N_2
Poison added		—	—	0.2×10^{-4} M dinitrophenol
	Time	Rate of pressure changes in $\frac{\text{mm.}}{\text{10 min.}}$		
Dark	40 min.	−0.3	+1.5	+0.7
Light. 1000 lux				
	First 10 min.	−18	+7	+9
	After 20 min.	−27	+3.5	+8
	After 50 min.	−26	+4	+7
Dark	10 min.	−1	+1	±0

going on in the unpoisoned algae. In the absence of carbon dioxide only the first effect becomes apparent.

It has been shown that photoreduction as well as normal photosynthesis are inhibited by dinitrophenol (Paper I). This substance is probably a specific poison for the transfer of hydrogen to carbon dioxide. That gives us an ex-

TABLE XVIII

Increase of Lactic Acid Formation in Presence of a Concentration of Dinitrophenol Which Inhibits the Dark Hydrogen Fermentation

0.055 cc. of cells of *Scenedesmus* D_3 in 3 cc. of 0.05 M phosphate buffer. pH 6.2. Temperature: 35°. Gas phase: N_2. 0.2 cc. of 10 per cent KOH solution in side arms of vessels. Dinitrophenol added anaerobically after 3 hours dark incubation.

Condition	Observation period	Control	4×10^{-4} M dinitrophenol
		Hydrogen liberated in c.mm.	
(*a*) Photochemical (low intensity)	20 min.	9	14
(*b*) Fermentative (dark)	11 hrs.	88	0
		Lactic acid (expressed in c.mm. normal gas)	
(*c*) Lactic acid formation	15 hrs.	5	47

TABLE XIX

Effect of Dinitrophenol on the Hydrogen Production in Scenedesmus D_3

0.082 cc. of cells in 3 cc. of 0.5×10^{-2} M $KHCO_3$. Temperature: 25°. Gas phase N_2; 4 per cent CO_2. Vessels 2 and 4 have 0.2 gm. palladium black in side arm.

		1	2	3	4
Volume of vessels, *cc.*		18.0	18.2	18.6	18.4
Volume of liquid, *cc.*		3.2	3.2	3.7	3.5
K_{CO_2}		1.60	1.62	1.65	1.63
K_{H_2}		1.36	1.38	1.37	1.37
Side arm		—	Pd	—	Pd
Dinitrophenol		—	—	0.6×10^{-3} M dinitrophenol	0.6×10^{-3} M dinitrophenol
	Observation period	Rates of pressure changes in $\frac{\text{mm.}}{30 \text{ mm.}}$			
Dark	150 min.	+5	+4	+18	+18
Light	50 min.	−1.5	−3	+32	+18
		Gas exchange in 30 min. computed from above data			
Dark reaction		+6.5 c.mm. CO_2; +1.4 c.mm. H_2		+30 c.mm. CO_2; ± 0 c.mm. H_2	
Light reaction (corrected for dark reaction)		−11 c.mm. CO_2; +0.7 c.mm. H_2		± 0 c.mm. CO_2; +19 c.mm. H_2	
		Formation of free CO_2 and of acid in the course of 6 hrs.			
(*a*) Total CO_2		+113 c.mm.		+323 c.mm.	
(*b*) Loss of bicarbonate		40 c.mm. acid		253 c.mm. acid	
(*a*) − (*b*)		73 c.mm. free CO_2		70 c.mm. free CO_2	
(*c*) Lactic acid (expressed in c.mm. of gas equivalents)		13 to 15 c.mm. lactic acid		85 to 112 c.mm. lactic acid	

planation why in Table XVII the photochemical production of hydrogen in absence of carbon dioxide appears to be stimulated by dinitrophenol. The fermenting cell has continually some carbon dioxide at its disposal, in spite of the absorbing potassium hydroxide in the side arm of the vessel. These small amounts react with part of the hydrogen which becomes available for reduction by irradiation. Dinitrophenol may inhibit this reaction thus allowing more hydrogen to be developed.

Needless to say that very high concentrations of poison will tend to affect also the light reaction. At such high concentrations so many substances otherwise not toxic will interfere with the metabolism of plants that, generally, one cannot speak any more of the specific poisoning of a certain reaction.

DISCUSSION AND CONCLUSIONS

The occurrence of a hydrogen fermentation in some species of green algae is not surprising, once it has been shown that these species can utilize molecular hydrogen in light and dark reactions (1). It is known that hydrogenase systems in bacteria may work both ways and catalyze (as should be the case with a true catalyst) the uptake as well as the evolution of molecular hydrogen. Farkas and Farkas (33) have demonstrated that one function of a hydrogenase consists in establishing an equilibrium between molecular and bound atomic hydrogen. Without any metabolic process going on, preparations of resting *B. coli* caused the complete exchange of some deuterium gas against an equivalent amount of hydrogen that was present in abundance as water in the liquid phase. This agrees with our findings that in the dark the same algae release hydrogen from internal hydrogen donors in an atmosphere of nitrogen while they take up hydrogen in an atmosphere of hydrogen. Gaffron pointed out that the uptake of carbon dioxide without a corresponding production of oxygen by illuminated algal cells during the induction period after incubation in nitrogen should be regarded as a reaction with internal hydrogen donors quite similar to the photoreduction with free hydrogen. The inhibition of the reduction with hydrogen by added glucose or yeast extract (1) was explained accordingly not as an inhibiting but as a competitive effect. Our results not only support this view but suggest that, as far as the reduction of carbon dioxide in the light is concerned, there cannot be any distinction between the hydrogen originating from molecular hydrogen or from internal hydrogen donors, since the latter are in equilibrium with the former. Another question is that of the pathway of the reducing hydrogen from the donor to the photochemical mechanism. In the case of internal hydrogen donors, the hydrogenase is possibly not included in the mechanism of photoreduction. A comparison of reaction velocities and studies with algae like *Chlorella* which lack the hydrogenase system might decide this question.

So far no essentially new problems have arisen from the experiments de-

scribed in Part I of this paper. The situation is different, however, if we turn to the photochemical evolution of hydrogen in Parts II and III. This reaction is not only new but unique. No analogy to it has been reported even among the many reactions of the purple bacteria, the place where it might have been found. The essential characteristics of the new reaction are as follows:

1. It is not an acceleration of a dark fermentation.
2. Its magnitude depends on the amount of internal hydrogen donors.
3. It appears in place of a carbon dioxide reduction whenever this reduction is inhibited either by actual absence of the substrate or by poisoning.
4. It shares with fermentation, photoreduction, oxyhydrogen reaction, etc., of these algae the property that it cannot be observed under aerobic conditions or under anaerobic ones when the proper reduction of the essential enzyme system has been blocked by oxygen or poisons.

To the question about the origin of the hydrogen produced photochemically, one may give at present more than one answer. One may, for instance, assume a direct photochemical decomposition of hydrogen donors, a kind of a "photofermentation" as it were. We prefer, however, the following more adequate hypothesis. We assume that the hydrogen originates from water in the photochemical reaction; that it is part of the hydrogen destined to reduce carbon dioxide. Unable to reach the substrate, the hydrogen is transferred to the hydrogenase and there transformed into the free gas. The dependency of this reaction upon the presence of suitable hydrogen donors in the cell becomes understandable if we consider that the photochemical production of reducing substances from water demands an equivalent formation of oxidized counterparts. In case the reducing substances are not used in the reaction with carbon dioxide, they will most likely form water again with those oxidized partners. Only inasmuch as the latter are reduced and taken care of by other reducing compounds (hydrogen donors) in the cell there is an opportunity for hydrogen made available by the photochemical reaction to be released as free gas by way of the hydrogenase system.

SUMMARY

1. After 2 hours of fermentation in nitrogen the metabolism of those algae which were found capable of photoreduction with hydrogen changes in such a way that molecular hydrogen is released from the cell in addition to carbon dioxide.

2. The amount of hydrogen formed anaerobically in the dark depends on the amount of some unknown reserve substance in the cell. More hydrogen is formed in presence of added glucose, but no proportionality has been found between the amount of substrate added and that of hydrogen formed. This is probably due to the fact that two types of fermentation reactions exist, with little or no connection between them. Whereas mainly unknown organic

acids are formed during the autofermentation, the addition of glucose causes a considerable increase in the production of lactic acid.

3. Algae which have been fermenting for several hours in the dark produce upon illumination free hydrogen at several times the rate observed in the dark, provided carbon dioxide is absent.

4. Certain concentrations of dinitrophenol strongly inhibit the evolution of hydrogen in the dark. Fermentation then continues mainly as a reaction leading to lactic acid. In such poisoned algae the photochemical liberation of hydrogen still continues.

5. If the algae are poisoned with dinitrophenol the presence of carbon dioxide will not interfere with the photochemical evolution of hydrogen.

6. The amount of hydrogen released in this new photochemical reaction depends on the presence of an unknown hydrogen donor in the cell; it can be increased by the addition of glucose but not in proportion to the amount added.

7. The results obtained allow for a more correct explanation of the anaerobic induction period previously described for *Scenedesmus* and similar algae. The possibility of a photochemical evolution of hydrogen had not been taken into account in the earlier experiments.

8. The origin of the hydrogen released under the influence of light is discussed.

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